



# Genetic Polymorphism of ACE (I/D) is Associated with Diabetic Nephropathy in Pakistani Subjects

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## ABSTRACT

Diabetic nephropathy (DN), also known as diabetic kidney disease (DKD), is a leading cause of morbidity and mortality in diabetic patients. A major cause of DKD is high blood pressure (hypertension) which remains uncontrolled chronically. Renin angiotensin aldosterone system (RAAS) is a major regulator of the blood pressure, electrolytes and fluid homeostasis. RAAS plays a key role in modulating the sodium metabolism, vasoconstriction, vascular tone and renal hemodynamics. Angiotensin converting enzyme (ACE) is a key member of RAAS pathway and target for anti-hypertensive drugs. ACE (I/D) polymorphism studies for its association with kidney function and risks of DN have been reported in different populations but with varying results. Hence, the objective of current study was to study the association of ACE (I/D) polymorphism with risk of DN in Pakistani subjects. For this study, 702 subjects were recruited who were divided into three groups; healthy control (n=222), diabetics without nephropathy (n=230) and diabetics with nephropathy (n=250). Clinically important biochemical parameters including glucose, uric acid, urea, creatinine, albumin, total protein cholesterol, and liver enzymes (ALP, ALT, and AST) were measured for all subjects. Genotyping for ACE (I/D) polymorphism was done by PCR assay. Statistical analysis showed that subjects with DN had higher (67%) frequency of ID genotypes and the ID genotype carriers also exhibited higher levels of creatinine (6.2±3.9 mg/dL) and urea (99±41 mg/dL). Moreover, logistic regression analysis also indicated that ACE ID genotype carriers had a 2.5-fold higher risk of DN as compared to the II genotype carriers. Hence the present study shows a strong association (OR 2.53, CI: 1.65-3.88, p<0.001) between ACE (I/D) polymorphism and DN in Pakistani subjects. Genetic polymorphism of ACE (I/D) is associated with diabetic nephropathy in Pakistani subjects. ACE ID genotype might also be considered as an important factor in risk stratification of the renal function for DKD and high blood pressure patients.

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## Authors' Contribution

WAK and KS designed and supervised the study. UAD and MH carried out the experimental work and written manuscript. BA helped with statistical analysis. FRA supervised the experimental work and helped in critical review of manuscript along with WAK and KS.

## Key words

Diabetic nephropathy, ACE (I/D) polymorphism, Hypertension, Renin angiotensin aldosterone system

## INTRODUCTION

Diabetic nephropathy (DN), also known as diabetic kidney disease (DKD) is considered as a debilitating health condition with high morbidity and mortality in diabetic patient (Gross *et al.*, 2005). DKD is characterized by

increased arterial blood pressure, elevated albuminuria and a gradual decline in glomerular filtration rate (GFR). Due to poor glycemic control in many diabetic patients, the function of kidneys gradually decline to cause pathological consequences (De Boer *et al.*, 2011). The main alteration in the kidney is the renal hypertrophy, which includes enlargement of glomerular size, thickening of glomerular basement membrane (GMB), mesangial expansion and inflammation which further leads to glomerular sclerosis and tubulointerstitial fibrosis. All such events ultimately lead to the end stage renal disease (ESRD) (Zain *et al.*, 2017; Tervaert *et al.*, 2010). Further pathophysiological changes which occur along with renal fibrosis are renal hemodynamic changes that cause afferent arteriolar dilation and efferent arteriolar constriction that leads to glomerular hypertension. It also causes oxidative stress and glucose

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metabolism abnormalities, inflammatory processes and overactive renin angiotensin aldosterone system (RAAS) that causes the pathogenesis of DKD (Hollenberg *et al.*, 2003; Wijnhoven *et al.*, 2007; Kobori *et al.*, 2013; Zain *et al.*, 2014). RAAS regulates blood pressure, electrolyte and fluid homeostasis in the circulatory system. This system also plays a key role in modulating the sodium metabolism, vasoconstriction, vascular tone, renal hemodynamics (Prabhakar, 2004). Genetic variants of the components of RAAS have been extensively studied relative to its biological and biochemical functions. Each of the RAAS components plays an important role in regulating the blood pressure and maintaining the normal function of kidney. Main components of RAAS are renin, angiotensinogen, angiotensin converting enzyme (ACE) and aldosterone synthase. Renin is synthesized in the juxtaglomerular cells during salt reabsorption, water retention, or sympathetic nervous system activation (te Riet *et al.*, 2015). Liver secretes inactive angiotensinogen protein, which is cleaved off by the renin and 10 amino acid long inactive peptide angiotensin I is generated. Angiotensin I is further converted to an eight amino acid long bioactive peptide called angiotensin II by the action of ACE which is secreted from the pulmonary and renal endothelial cell. Effects of angiotensin II are regulated by specific cell surface receptors, angiotensin type 1 (AT1) receptor and angiotensin type 2 (AT2) receptor, which are mainly expressed in the kidney, adrenal glands, heart and vascular smooth muscle (Jan Danser *et al.*, 2007). Moreover, ACE also plays role in Bradykinin-Kallikrein pathway and catalyzes the breakdown of bradykinin (a vasodilator) into inactive peptide fragments. Deregulation of RAAS system plays a crucial role in the progression of diabetic nephropathy. Mainly, RAAS enhances the intraglomerular pressure as well as causes the diffused diabetic glomerulosclerosis (Putnam *et al.*, 2012; Hussain and Awan, 2018). Various studies have reported that polymorphisms of *ACE* can influence the kidney function. Till date, approximately 160 genetic variants have been identified in the *ACE*. Among these insertion/deletion (I/D) is the most widely studied polymorphism. *ACE* (I/D) polymorphism is an insertion or deletion of 287 bp *Alu* repeats which are located in the intron 16 of *ACE*. Although it is located in non-coding region, its functional studies have shown that this polymorphism affects the activity of the promoter region and influence the expression of *ACE* gene. It has been reported that deletion (DD) polymorphism leads to increased *ACE* enzyme synthesis and its function (Berl, 2009); as a result the risk of coronary heart diseases will increase in diabetic and non-diabetic patients. Genome wide association studies have shown that individuals with DD genotype have higher levels of *ACE* enzyme, while

II genotype decreases the *ACE* levels. The concentration of *ACE* enzyme in subjects with ID genotype was intermediate as compared to II or DD genotype carriers. Balance in *ACE* concentration and activity of RAAS pathways is very critical for maintaining kidney function. Therefore, an imbalance in *ACE* secretion is associated with susceptibility to renal hemodynamics which promotes progressive renal injury and can cause renal tubular dysgenesis which can further lead to glomerular sclerosis. During the progress and development of DN, persistent high blood sugar along with low glomerular filtration rate leads to renal inflammation, diffused glomerulosclerosis and tubulointerstitial fibrosis subsequently causing kidney failure (Hussain *et al.*, 2020, Ilić *et al.*, 2014). Since limited research on *ACE* (I/D) polymorphism has been done in Pakistan, the main objective of the current study was to study the genotypic frequencies of *ACE* (I/D) polymorphism in Pakistani subjects and to investigate the association of *ACE* (I/D) polymorphism with clinically important biochemical parameters and risk of diabetic nephropathy.

## MATERIALS AND METHODS

### *Subject selection and sample collection*

The study subjects have been categorized into three groups as: (i) Healthy subjects (C; n=222) who had no family history of diabetes or diabetic nephropathy, Samples from control subjects were collected from peripheral areas of Sargodha and Faisalabad districts of Punjab, Pakistan. (ii) Subjects with diabetes (D; n=230) but without nephropathy were recruited from the Allied Hospital, Faisalabad and Sadiq Hospital, Sargodha, Pakistan. (iii) Subjects having diabetes as well as nephropathy (DN; n = 250) were enrolled from the Dialysis Unit, Allied Hospital, Faisalabad, Pakistan and Chiniot Dialysis Center, Faisalabad, Shalimar Hospital Lahore, and District Headquarter Hospital (DHQ) Lahore, Pakistan.

All subjects were above 35 years and comprised of both genders. Prior to sample collection, approval was taken from the institutional ethics research committee and written informed consent was taken from all participants of the study. The demographic (name, age, gender, education, address, nutritional and personal habits), anthropometric [Body Mass Index (BMI)] and clinical (disease history, blood pressure etc.) details were taken on a pre-designed questionnaire form.

Six mL venous blood sample was taken from all subjects, 3 mL was taken in EDTA vacutainer for genomic DNA extraction and rest of the blood was used for serum biochemical analysis.

### Biochemical analysis

For biochemical analysis, serum was separated from the blood samples by centrifugation. All biochemical parameters such as glucose, albumin, total protein, uric acid, urea, creatinine, cholesterol, triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) were measured on semi-automated clinical chemistry analyzer (MicroLab 300, Merck) as reported in our previous papers (Hussain and Awan, 2018).

### Genotyping of ACE (I/D) polymorphism

Genetic analysis was performed as reported in our previous papers (Hussain *et al.*, 2020). Briefly, genomic DNA was extracted from all blood samples by organic method. Quantity of extracted DNA was measured by nanodrop and DNA was stored at -20 °C until further analysis. Genomic DNA from all subjects were amplified by polymerase chain reaction (PCR) using previously reported (Hussain and Awan, 2018), oligonucleotide primers for the target fragment in the *ACE*. Forward primer: 5' CTGGAGACCACTCCCATCCTTTCT 3'

Reverse primer: 5' GATGTGGGCATCACATTCGTCA 3'

Thermal cycler profile for PCR reaction was: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min of denaturation, primer annealing at 59°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min in T100™ thermal cycler (Bio-Rad Laboratories, Inc.). PCR reaction mixture of 30 µL had 100–200 ng/µL of genomic DNA, 3 µL 10X PCR buffer (750 mM Tris-HCL, pH 8.8), 1.5 mM MgCl<sub>2</sub>, 0.12 mM dNTPs, 0.2 µM of each primer, 1.6 U Taq polymerase (Fermentas), and 19.9 µL water. The amplified product was run on 1.2% agarose gel to which ethidium bromide was already added. 1 kb ladder was run along the samples for size estimation and the gel was visualized under UV light in Gel Doc™ EZ System (Bio-Rad Laboratories, Inc.). DD genotype showed band at 190 bp, II genotype showed band at 490 bp while ID genotype showed both bands at 190 and 490 bp. In ID genotype carriers, D allele is preferentially amplified and mask the amplification of I allele. Thus, DD genotype carriers were subjected to another round of PCR with I-allele specific primer. Amplification of 337bp product showed the presence of I allele and ID genotype.

### Statistical analysis

Statistical analyses were performed by using SPSS version 20 (IBM Inc.). Student's t-test was applied for comparison of clinical and biochemical parameters (as Mean ± S.D) of the study groups (C, D, DN). Gene counting method was used to calculate the allelic frequencies while

chi-square ( $\chi^2$ ) test was used to test for the association of genotypes (II, ID, DD) and alleles (I, D) with diabetes and diabetic nephropathy. ANOVA was used to see the association of clinical and biochemical parameters with *ACE* I/D polymorphism in the study population. Moreover, logistic regression analysis was performed to find the disease risk associated with genotypes.

## RESULTS AND DISCUSSION

### Demographic data of study subjects

Table I shows the demographic, clinical and biochemical features of all three study groups. SBP was significantly increased in subjects with diabetes as compared to healthy controls, however no significant increase in DBP was observed. Glucose level is significantly higher in diabetic subjects relative to healthy control subjects as expected. Interestingly, the maximum increase in concentration of total protein was observed for C, however, the concentration of albumin was higher in DN group. Liver function test shows substantial increase in the levels of ALT, ALP in DN patients, in contrary to this AST values showed no significant difference in all three study groups. Similarly, cholesterol and triglyceride also showed considerable difference in all study groups. Kidney function test has shown significant increase in serum creatinine (5.14±2.99 mg/dL) in DN as compared to C and D, similarly DN group has highest value of urea and uric acid that evidence to statistically significant.

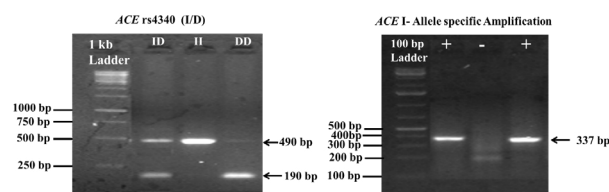


Fig. 1. Genotyping of ACE (I/D) polymorphism by PCR.

### Genetic analysis

Genotypes for *ACE* (I/D) polymorphism include II, ID and DD (Fig. 1). Statistical significance was tested for both alleles (I/D) and all three genotypes by chi-square test (Table II). Significant differences were observed in frequencies of II, ID, and DD genotypes among C, D and DN groups. ID genotype was found frequent in DN patients (67%) followed by C (50%) and D (49%) subjects. Chi-square test also showed that ID genotype ( $\chi^2=25.06$ ,  $p<0.001$ ) is strongly associated with the increased risk of DN. Similarly, gene counting method was used to calculate the allelic frequencies of I and D alleles in all study groups.

**Table I. Demographic, clinical and biochemical features of study subjects.**

Characteristics	C (n = 222)	D (n = 230)	DN (n = 250)	C vs DN (p value)	D vs DN (p value)
Age (years)	42±8	55±10	55±7	-	-
SBP (mmHg)	117±22	140±26	149±22	<0.001	0.06
DBP (mmHg)	77±11	88±16	87±12	0.995	<0.001
Glucose (mg/dL)	93±25	191±110	151±57	<0.001	<0.001
Albumin (g/dL)	4.04±0.32	4.06±0.78	4.17±1.27	<0.001	<0.001
Total protein (mg/dL)	7.20±1	6.27±0.99	6.75±1.95	<0.001	<0.001
ALT (U/L)	26±18	27±16	36±21	<0.001	<0.001
AST (U/L)	30±17	33±20	33±23	0.138	0.943
ALP (U/L)	159±58.71	238±124.18	288±126.13	<0.001	<0.03
Creatinine (mg/dL)	0.96±0.25	1.14±1.0	5.14±2.99	<0.001	<0.001
Urea (mg/dL)	26±9	42±32	103±45	<0.001	<0.001
Uric acid (mg/dL)	5.90±1.82	6.83±3.05	7.63±3.05	<0.001	0.87
Cholesterol (mg/dL)	186±45	191±59	315±165	<0.001	<0.001
Triglycerides (mg/dL)	259±131	214±85	222±146	0.983	<0.001

C, Healthy control subjects with no diabetes or diabetic nephropathy; D, Subjects with diabetes but no diabetic nephropathy; DN, Subjects with diabetes and diabetic nephropathy; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase, ALP, Alkaline phosphatase Significant difference for † (C vs D) and \* (D vs DN).

Allelic frequencies of *ACE* (I/D) polymorphism revealed that in diabetic subjects I allele is more frequent than D allele (I: 62% vs D: 38%) as compared to the control subjects ( $p < 0.038$ ,  $\chi^2 = 6.56$ ) (Table II).

One-way ANOVA was used to find the association of clinical and biochemical parameters with genotypes of *ACE* (I/D) polymorphism in the study population. Statistical results showed that the subjects carrying *ACE* ID genotype have higher concentration of creatinine and urea in comparison to the II and DD genotype, however, the difference among the *ACE* (I/D) genotypes is not statistically significant (Table III).

**Table II. Allelic and genotypic frequencies of ACE (I/D) gene polymorphism.**

Polymorphism [ACE (I/D)]	C	D	DN	Significance
<b>Genotype</b>				
II	67(30%)	82(37%)	50(20%)	$\chi^2 = 25.06$ , $p < 0.001$
ID	111(50%)	108(49%)	167(67%)	
DD	44(20%)	31(14%)	32(12.9%)	
<b>Allele</b>				
I	245(55)	272(62%)	267(54%)	$\chi^2 = 6.56$ , $p < 0.038$
D	199(45)	170(38%)	231(46%)	

C, Healthy subjects with no diabetic nephropathy and no diabetes; D, Individuals with diabetes but no diabetic nephropathy; DN, Subjects with Diabetic nephropathy and diabetes.

Logistic regression analysis showed that ID genotype increase the risk of DN by 2.5 times [OR 2.53, CI: 1.65-3.88,  $p < 0.001$ ]. In contrary to this, ID genotype had showed no significant difference in diabetic subjects [OR 0.79 (CI: 0.52–1.21)  $p = 0.28$ ] (Table IV).

Major findings of this study demonstrate that serum urea and creatinine levels were significantly higher in DN patients as compared to other groups which validate the selection of patient group. Genetic analysis for *ACE* (I/D) polymorphism also showed that the frequency of ID genotype carrying subjects is more in DN group as compared to diabetic and healthy control groups. Moreover, logistic regression analysis also demonstrated that ID genotype increase the risk of DN by 2.5-fold. Along with environmental factors, co-occurring diseases and many genetic factors, genetic variants of RAAS genes influence the risk of DN. RAAS has various subsystems which contribute to the disease pathology. RAAS starts with the release of renin enzyme which cleaves off 10 amino acids peptide from the C-terminal of the angiotensinogen (inactive protein) that is then called as angiotensin I (Ang I; inactive). ACE cleaves off further 2 amino acids from Ang I and converts it to a bioactive octapeptide as angiotensin II (Ang II). Conversion of Ang I to Ang II is a rate limiting step in the regulation of RAAS. Ang II acts as vasoconstrictor and increase the arteriolar blood pressure which leads to thickening of the GBM, mesangial cell hypertrophy, increased mesangial extracellular matrix and reduced

**Table III. Association of clinical and biochemical parameters with ACE I/D polymorphism.**

Parameter	Group	ACE (I/D) polymorphism			Significance
		II	ID	DD	
SBP (mmHg)	C	118±13	115±14	121±15	0.14
	D	136±28	142±27	144±24	0.39
	DN	148±24	149±21	152±31	0.72
DBP (mmHg)	C	79±11	76±11	80±10	0.17
	D	86±15	90±16	92±18	0.25
	DN	89±11	87±13	87±12	0.52
Glucose (mg/dL)	C	97±32	93±24	85±12	0.06
	D	187±106	199±119	176±90	0.55
	DN	158±87	148±48	164±58	0.42
Uric acid (mg/dL)	C	5±2	6±2	6±2	0.13
	D	6.4±2.8	7.1±3.3	6.9±2.6	0.23
	DN	7.7±3.1	7.5±3.1	8.2±2.9	0.64
ALT (U/L)	C	28±19	27±18	26±16	0.86
	D	26±16	28±17	27±19	0.88
	DN	39±22	36±21	36±23	0.89
AST (U/L)	C	32±18	30±17	29±17	0.78
	D	34±21	35±22	30±14	0.39
	DN	34±19	33±25	32±18	0.97
ALP(U/L)	C	167±62	159±61	152±49	0.46
	D	249±139	234±113	228±121	0.62
	DN	259±98	291±133	311±118	0.38
Albumin (g/dL)	C	4.04±0.27	4.03±0.37	4.09±0.29	0.55
	D	4.1±0.8	4.0±0.7	4.3±0.8	0.27
	DN	4.3±1.4	4.2±1.2	4.1±1.4	0.88
Totl Protein (mg/dL)	C	7.24±0.94	7.27±1.01	6.95±1.09	0.197
	D	6.2±1.0	6.3±0.9	6.3±1.02	0.52
	DN	6.4±1.7	6.8±2.1	6.9±1.6	0.64
Creatinine (mg/dL)	C	0.91±0.29	1.01±0.21	0.93±0.26	0.023*
	D	1.2±0.7	1.3±1.9	1.1±0.8	0.40
	DN	4.9±2.8	6.2±3.9	5.1±2.9	0.18
Urea (mg/dL)	C	28±10	26±9	27±8	0.42
	D	39±26	45±36	42±31	0.47
	DN	112±55	99±41	117±52	0.14
Cholesterol (mg/dL)	C	191±53	186±41	175±40	0.21
	D	196±63	188±59	193±55	0.63
	DN	303±183	306±149	377±213	0.19
Triglyceride (mg/dL)	C	279±134	258±143	235±94	0.22
	D	216±89	217±88	202±59	0.68
	DN	213±114	225±158	220±124	0.93

C, Healthy subjects with no diabetic nephropathy disease and no diabetes; D, Individuals with diabetes. DN, Subjects with diabetic nephropathy and diabetes. SBP, Systolic blood pressure; DBP, Diastolic blood pressure; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; ALP, Alkaline phosphatase; \* shows no statistically significant difference is found between genotype and biochemical parameters in study groups.

**Table IV. Logistic regression analysis for the ACE genotype associated risk of diabetes and diabetic nephropathy.**

Disease	ACE (I/D) polymorphism		
	DD	ID	II
Diabetic Nephropathy <sup>*</sup>	1.69 (0.923-3.104) 0.089	2.53 (1.65-3.88) p<0.001*	Ref
Diabetic <sup>^</sup>	0.576 (0.328-1.00) 0.054	0.795 (0.524-1.207) 0.282	Ref

Diabetic vs. diabetic nephropathy <sup>^</sup>, \* shows statistical significance. OR, odds ratio; CI, confidence interval; Ref, Reference.

Diabetic Nephropathy<sup>\*</sup>, comparison between diabetic and diabetic nephropathy; Diabetic<sup>^</sup>, comparison between control and diabetic.

podocyte number. Ang II also stimulates inflammation, cell apoptosis, migration and differentiation. Monocyte chemoattractant protein-1 production responsible for renal fibrosis is also regulated by RAAS. Various studies have shown that polymorphisms of ACE gene significantly influence the biological functions of RAAS and thus could influence the occurrence and progression of DN.

DN is a major complication of diabetes and is characterized by proteinuria, reduced GFR and disrupted metabolism. Previous studies showed that DN has become the primary cause (25-47%) of end-stage renal disease (ESRD) in Japan, India, Europe and United States (Rahimi *et al.*, 2011). Different factors including environmental constituents, genetic variants, various growth factors, inflammatory mediators and cytokines contribute to the impairment of renal functions in diabetes.

Similar to the results of the current study, the studies from Japanese and south Indian population shows that ACE (I/D) polymorphism influences the onset of DN. However, various reports from Iranian and Malaysian population did not show any association between ACE (I/D) polymorphisms and DN. Association between ACE I/D polymorphism and DN was also reported in French people and this polymorphism was considerably linked with higher risk of DN in large population of France, Denmark and Finlan (Cambien *et al.*, 1992) Furthermore, in some patients from the capital city of Iran (Tehran), the presence of D allele was associated with enhanced ACE activity resulting in higher albuminuria, while no association of ACE D allele was reported for populations of Europe, Western Asia, Central Asia, South Asia and North Africa. Moreover, in Asian Indian and Tunisian the ratio of D allele DD genotype in patients having DN is considerably high as compared to the patient having

diabetes but no nephropathy; however, these findings contradict the results of current study which favors the *ACE* ID genotype for increasing the risk of DN (Börgeson and Godson, 2010).

Our study is supported by the results of a systematic review comprises of over 47 studies from 1994 to 2004, which states that ID genotype is predominant in patients than the healthy control subjects. Association between ID genotype and diabetic nephropathy disease has also been observed in North Indians (Brownlee, 2005). Thus, it could be suggested that ID genotype increases the risk of DN by increasing *ACE* activity, which will eventually lead to increased production of Ang II. As Indian population is very close to the Pakistani population in their genetic makeup, culture, dietary habits and physical activity, however, the results from Indian population for the association of *ACE* (I/D) polymorphism with DN are inconclusive. Some studies from south Indian population showed the association of D allele of *ACE* polymorphism with DN (Viswanathan *et al.*, 2001; Ismail *et al.*, 2004), while other studies from same population reported no association between *ACE* (I/D) polymorphism and DN (Golmohamadi *et al.*, 2006; Kumar *et al.*, 2013). This contradiction in results could be due to the heterogenous sample size and inclusion criteria. Association of *ACE* (I/D) polymorphism with DN has also been studied in Pakistani population; however, the results are conflicting. In 2005, it was studied that 110 DN subjects and concluded that D allele of *ACE* (I/D) polymorphism acts as genetic risk factor for DN (Kumar *et al.*, 2005). In contrast to this, another group studied 51 DN subjects and reported that *ACE* (I/D) polymorphism is not associated with DN (Prasad *et al.*, 2000). Contrary to both of these studies, current study on 250 DN subjects reports that ID genotype increase the risk of DN by 2.5-fold. To resolve this contradiction, studies with larger sample size and more stringent criterion are needed.

## CONCLUSION

Carriers of *ACE* ID genotype have increase concentration of creatinine and urea. *ACE* ID genotypes carriers also have 2.5 folds higher risk of diabetic nephropathy as compared to the subjects with II genotype.

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### IRB approval

This study was approved by Institutional Review Board, University of Sargodha, Sargodha on 21-05-2018.

### Ethical approval

This study was approved by the Institutional (National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan) Ethics Review Committee.

### Statement of conflict of interest

The authors have declared no conflict of interest.

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